



PERSPECTIVE

ELECTROPORATION: BIO-ELECTROCHEMICAL MASS TRANSFER AT THE NANO SCALE

Rafael Davalos, Yong Huang, and Boris Rubinsky

*Biomedical Engineering Laboratory, Department of Mechanical Engineering,
University of California at Berkeley, Berkeley, California, USA*

This article provides a brief review of the field of electroporation and introduces a new microdevice that facilitates studies to test theories, gain understanding, and control this important biomedical technology. Electroporation, a bio-electrochemical process whose fundamentals are not yet understood, is a means of permeating the cell membrane by applying a voltage across the cell and forming nano-scale pores in the membrane. It has become an important field in biotechnology and medicine for the controlled introduction of macromolecules, such as gene constructs and drugs, into various cells. It is viewed as an engineering alternative to biological techniques for the genetic engineering of cells.

To study and control electroporation, we have created a low-cost microelectroporation chip that incorporates a live biological cell with an electric circuit. The device revealed an important behavior of cells in electrical fields. They produce measurable electrical information about the electroporation state of the cell that may enable precise control of the process. The device can be used to facilitate fundamental studies of electroporation and can become useful in providing precise control over biotechnological processes.

INTRODUCTION

Definition and Description

The cell membrane is made of a lipid bilayer that separates the interior of the cell from the exterior. The function of a cell membrane is to isolate between the chemical environment in the interior of the cell from the chemical environment outside the cell and to allow only membrane-selected chemical species to pass through. However, in many biotechnological and medical applications it is important to introduce into cells chemicals that would normally not pass the cell membrane. This includes gene constructs in genetic engineering of cells, or drugs in treatment of diseases. Electroporation is a phenomenon that makes cells porous by exposing them to strong, quick electric pulses [1]. It is thought that the electrical field changes the electrochemical potential around a cell membrane and induces instabilities in the polarized cell membrane lipid bilayer. The unstable membrane reverts shape to form aqueous nano-scale pores through the membrane [2]. Mass transfer

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Address correspondence to Boris Rubinsky, Biomedical Engineering Laboratory, Department of Mechanical Engineering, University of California at Berkeley, Berkeley, CA 94720. E-mail: rubinsky@newton.berkeley.edu

NOMENCLATURE

a	cell radius, m	V_m	transmembrane potential (potential difference), V
C_m	membrane capacitance per unit area, F/m ²	θ	azimuthal angle, rad
E_o	applied electric field, V/m	λ	cell geometry shape factor
G_m	membrane conductance per unit area, S/m ²	σ_e	extracellular conductivity, S/m
I_{ep}	electroporation current, A/m ²	σ_i	intracellular conductivity, S/m
I_{ion}	ionic current, A/m ²	τ	time constant, s ⁻¹
t	time after constant field is turned on, s	Φ_e	extracellular potential, V
		Φ_i	intracellular potential, V

can now occur through these pores under electrochemical control. The technique works for both mammalian and plant cells. Although it is used extensively, electroporation is a relatively new science, and the fundamental biophysics of electroporation are not completely understood [1].

To be useful, the process of electroporation must be controlled and reversible. The reversibility is a function of ionic strength of the suspending medium; temperature; electrical pulse voltage parameters—amplitude, length, shape, and repetition rate; as well as cell type and development stage [3, 4].

Current Applications

This process is used in many in-vitro and in-vivo applications at the cellular level and the whole tissue level. Current applications include:

- Gene transfer in mammalian cells
- Genetic manipulation in plant cells
- Genetic transformation of bacteria and yeast
- Introduction of compounds into cells
- Cell loading
- In-vitro and in-vivo drug delivery
- Production of hybridoma and human monoclonal antibodies
- Embryo cloning, electroinsertion of proteins into cell membranes [2].

It is anticipated that electroporation will become an important technology in the treatment of genetic diseases at the single-cell level, in particular with stem cells, such as in treatment of autoimmune diseases and hemophilia, and at the whole-tissue level, such as in treatment of cystic fibrosis or diabetes. Furthermore, electroporation is viewed as a method for the treatment of cancer through the introduction of drugs into malignant cells.

General Procedure

A typical experiment involves placing a sample between two electrodes and applying a number of quick high-voltage pulses to it. Figure 1 illustrates the process of electroporation. The cell subjected to a pulsed electric field becomes permeable at the locations with the largest transmembrane potential, the areas closest to the electrodes.

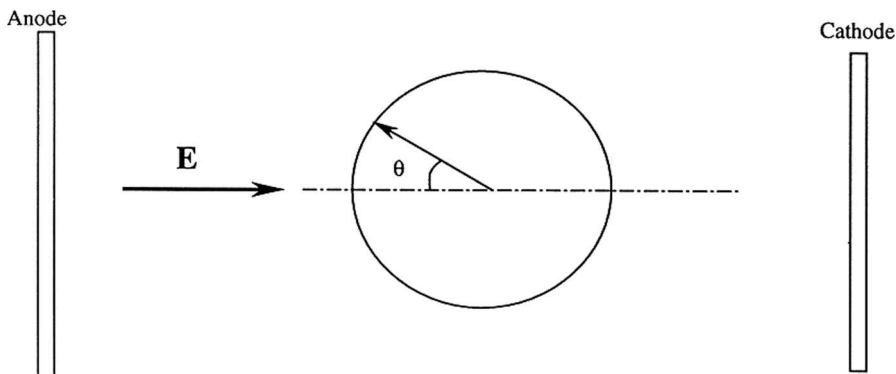


Figure 1. The model of a cell in an electrical field.

The electrical field is pulsed, usually DC rectangular or exponentially decaying, in order to prevent irreversible cell damage [2]. A rectangular pulse can be generated using a high-voltage power supply, while an exponential pulse is generated by discharging a capacitor.

When dealing with a population of cells, there is a trade-off between cell viability and transfection efficiency. For instance, in electrochemotherapy (the introduction of chemotherapeutic drugs into cells through electroporation), tumors are treated with Bleomycin, typically at 1,300–1,500 V/cm, width 100 μ s, frequency 1 Hz, using 8 pulses in one direction [5]. Because of the lack of fundamental understanding of the biophysics of electroporation, it is difficult to determine the parameters for electroporation or to study the phenomenon. For instance, experimenters estimate the pore size based on the size of the molecules that were introduced into the cell.

To obtain more insight of this phenomenon, numerous empirical studies have been conducted. They varied voltage parameters such as amplitude, frequency, pulse number, pulse type, number of pulses, and electrode geometry and studied the consequence on cell viability or its ability to incorporate molecules. Procedures for selecting values for the electrical parameters are described by Chang et al. [2], who provide other research as reference points. Some of the scenarios studied included the skin of cadavers, liver cells and brain tumors in rats, and embryonic chicken hearts, just to name a few. Table 1 provides a brief list of some of the more current studies.

Advantages and Disadvantages

The abundant advantages of electroporation make this technique very attractive: it is technically simple; it can be used to treat a whole population of cells; it has a broad application for the transfer of any macromolecule; it provides greater efficiency of transfection for some cell lines; and it can be applied equally successfully to prokaryotic and eukaryotic cells without major modifications and adaptation to cell type and origin [6, 7]. With respect to gene transfer, since electroporation is a nonviral form of gene transfer, it has a variety of advantages over the use of viral vectors, which is a major biochemical technique to insert genes by means of virus vessels. Some of the advantages are: any type of cell or tissue can be a target; cell handling is quick and easily done;

Table 1. Electroporation parameters from different researchers

Cell type	Molecules	Pulse conditions	Analysis and detection method	References
Rat muscle fiber and skeletal muscle	Genes	530 V/cm, 99 μ s, 8 pulses	FDM; Cr(51)-EDTA	Gehl and Sorensen [8]
Skin of hairless rats		100 V, 500 ms, 5 pulses, 1 Hz		Reginer et al. [9]
Human cadaver skin	Vitamin C	60–100 V, 2.7–30 ms, 6 pulses	Computer simulation of electric field	Zhang et al. [10]
Corneal endothelium in rats	Genes	5–40 V/cm, 50 ms, 8 pulses	Color reaction x-gal-positive areas	Oshima et al. [11]
Embryonic chicken hearts	DNA	3–12 pulses	Green fluorescent protein	Harrison et al. [12]
Sperm of loach	Antifreeze protein	8,000 V, 0.12 ms, 2–8 pulses		Tsai and Tseng [13]
Tumors in mice	Bleomycin	1,040 V, 0.1 ms, 4–8 pulses	FEA of <i>e</i> -field, magnetic resonance CDI	Miklavcic [14]
Bovine adrenal medullary cells	Rb, Cr-EDTA	2 kV/cm, 200 μ s, 1–100 pulses		Baker and Knight [15]
Liver cells in rats	Genes	50 V, 50 ms, 8 pulses	Digital analysis of microscopic images	Suzuki et al. [16]
Human red blood cells		DC: 100 kHz, 0.3 ms, 5 kV/cm	Rapid-freezing electron microscopy	Chang et al. [2]
Human prostate adenocarcinoma cell		SP: 10–60 V, 60 ms	Transmembrane current	Huang and Rubinsky [17]

repeated administration of DNA is possible; no immunogenicity is expected; and there are no constraints on amounts and sizes of DNA to be used [4, 7, 8].

Since electroporation is relatively new, there are several problems to overcome to ensure that permeation of the membrane is controlled and reversible. For example, some electrical pulse parameters will not form any pores and the duration of the pulse required to permeate a cell is not much smaller than a pulse that would destroy the cell. While electroporation appears to be a universal phenomenon, the outcome of an electroporation protocol is cell type specific and varies among cells in a given population [18]. These issues become more complicated when dealing with whole tissues, especially *in vivo*, due to the large variation in tissue properties. While electroporation is viewed as an important technique for treatment of genetic diseases, predictive models and control mechanisms must be developed before this technique can be applied on human internal tissue *in vivo*.

BACKGROUND

History

Over the years, there has been a great deal of work in the field of electroporation. This section briefly highlights some of the key contributions to the field. Dielectric breakdown of the cell membrane due to an induced electric field was first observed in the early 1970s [2, 19, 20, 21]. Pore formation in the cell membrane and the ability to reseal these pores by actually pulsing this electric field were both discovered separately during the late 1970s [2, 15, 22, 23]. By varying parameters, this induced electric field would either slightly increase the conductivity of the membrane; cause incomplete reversible electrical breakdown; cause reversible electrical breakdown; or destroy the cell membrane [4, 24]. By the early 1980s, people began using this method to introduce compounds into individual cells and toward the late 1980s expanded the method to multicellular tissue [2].

In 1992 Chang et al. visualized electroporation of red blood cells using rapid-freezing electron microscopy and postulated that it is a dynamic process with three stages: pore formation; pore expansion; and pore resealing [2].

Okino [25] and Mir in 1993 [25, 26] demonstrated that electroporation aids in the treatment of tumors by enhancing chemotherapeutic drug delivery into the cytosol and termed the treatment Electrochemotherapy [5].

Muramatsu et al. [27], Zimmerman [28], Singh and Dwivedi [29], Ho and Mittal [4], and Neumann [30] provide good reviews of electroporation. Muramatsu et al.'s [27] review describes electroporation as a potentially powerful and convenient means for non-viral gene transfer to tissues *in vivo*. Singh and Dwivedi focus on tissue electroporation to enhance local delivery of chemotherapeutic agents to solid tumors.

Electroporation Theories

According to Ho and Mittal [4], the theoretical research can be broken down into four major areas: the transmembrane potential measurements; dynamics of electroporation, i.e., electropore formation sequence and pore growth rate, properties of electropores such as size, structure, and population; membrane permeabilization and breakdown theory; and the effects of secondary factors, such as temperature, ion type, chemicals, and cell growth stage, on electroporation.

Different approaches have been taken to describe the electroporation phenomena, such as energy balance theories, or electromechanical and stochastic pore population approaches, but none of them has been rigorously validated due to the deficiency of direct experimental results.

The early work was done by Crowley [19] who used electromechanical instability theory to describe membrane breakdown. The electrostatic compressing force, which balances the elastic counter force of the membrane, decreases membrane thickness. Assuming that the bilayer volume is incompressible, the membrane area must increase and this leads to an increased wedge-shaped conformation of lipids. The membrane breakdown then can be explained as a result of the destabilized bilayer due to the shift of phase equilibrium toward nonlamellar phases. Though supported by some experimental evidence, this theory cannot distinguish the reversible membrane breakdown and irreversible rupture of membrane. Bryant and Wolfe [31] argued that cell lysis was due to isotropic mechanical surface tension produced in deforming the cell rather than due to electric field produced in the membrane. Wilhelm et al. [32] incorporated ideas of pore formation as well as mechanical stress to be accounted for by membrane breakdown.

Researchers offered many theories and have speculated what in actuality are these pores and what cause them. Neumann and Boldt [33] explained them as just a phase transition in the lipid layers. On the other hand, Cheromordik [34] theorized that initially there are small hydrophobic pores or defects which becomes enlarged and hydrophilic by the electric field. Tsong [35] speculated that the membrane could either be permeated at the protein channels or on the lipid bilayers, but Neumann [36] suggested that permeation is confined primarily to the lipid bilayer. In 1989 Neumann [3] described permeabilization as more of an indirect effect of the electric field and that the structural changes of the membrane phase are preceded by the ionic interfacial polarization [4].

The microelectroporation device discussed later may provide a means for validating these theories that up until now have been subject to argument.

Current Research

This section describes some of the current work at various research institutions in the field. Due to the succinct nature of a journal article, this section is devoted primarily to research in the more theoretical aspects of electroporation. Because of their importance, the more empirical research has been included in Table 1.

Weaver describes electroporation at a more fundamental level, at the individual pores themselves. For example, they used transient aqueous pore theory to estimate the amount of the membrane which was opened. Weaver and Powell [37] offered two explanations for electroporeabilization: either the electric field quickly creates small pores in the membrane, or miniscule pores already exist in the membrane. The large potential then causes these pores to expand.

Weaver and Barnett [38], realizing that the external environment influenced the transmembrane potential and membrane conductance, developed models to predict the number of electropores and the pore population rate in a membrane. These pores would remain stable provided that energy was expended for edge formation and energy was gained by increasing the pore areas. Expanding their model, Freeman et al. [39] predicted that, at a transmembrane potential of 1 V, these pores constitute less than 0.1% of the membrane area [4]. A good introduction to their work on "reversible electrical breakdown" can be found in Chang et al. [2].

Gehl and Sorenson [8] looked at the in-vivo gene transfer of muscle fiber and skeletal muscle in rats by electroporation. The parameters optimized were electric field strength, pulse duration, and pulse number. For the skeletal muscle, the threshold for permeabilization was 0.53 kV/cm (± 0.03 kV/cm) using eight pulses of 99- μ s duration at a frequency of 1 Hz. The electric field distribution was calculated using a finite-difference technique and the equivalence principle (i.e., that the electric potential and electric charge are equivalent) at the boundary. Permeability was determined in vivo by measuring the uptake of the marker Cr(51)-EDTA. By subtracting the Cr(51)-EDTA content of nonelectroporated muscle from electroporated muscle in the same mouse, it is possible to determine the net internalization of Cr(51)-EDTA as a result of electroporation.

Miklavcic's work [5] is mentioned because it was the first work that appears to look beyond the typical empirical approach for in-vivo electroporation. The authors were striving for a more qualitative understanding of the phenomenon by evaluating and comparing different distributions of electric fields of tumors in mice. Their method was essentially a four-step process. First, design different electrode sets that produce notably different distributions of an electric field in the tumor. Then, qualitatively evaluate the current density distribution for the electrode sets by means of magnetic resonant current density imaging. Next, use a finite-element model to explain the observed effects, and the boundary element method to describe the e -field variation. And finally, demonstrate the difference in electrochemotherapy effectiveness for the different sets [5].

Miklavcic et al. observed that different electrode orientations yielded dramatically different results. Applying four pulses in one direction and then reorienting it in another direction produced much better results. They offered two explanations. At the macro level, at any particular orientation some cells would not permeate because of the variations in tissue electrical properties. At the micro level, they explained that the effect was due to cellular shape. Since cells are not ideally spherical, they may have more transmembrane potential with one orientation versus another. It should be noted that their 50-V model correlated well with the currents measured with the CDI; however, the 1,300-V model was 10 times less than the measured currents. The most probable explanation for this is that the conductivity of an electroporated cell is an order of magnitude higher than normal, and the model just assumed a constant conductivity of 0.27 Sm^{-1} for the tumor.

MATHEMATICAL MODELS

DeBruin and Krassowska [40] developed a macroscopic mathematical model of an isolated single cell exposed to an external electric field. Since both the intracellular and extracellular domains are source-free, the transmembrane potential is governed by Laplace's equation:

$$\nabla^2 \Phi_i = 0 \quad \text{in intracellular space} \quad (1)$$

$$\nabla^2 \Phi_e = 0 \quad \text{in extracellular space} \quad (2)$$

The current density across the membrane S is given by

$$-\mathbf{n} \cdot (\sigma_i \nabla \Phi_i) = \mathbf{n} \cdot (\sigma_e \nabla \Phi_e) = C_m \frac{\partial V_m}{\partial t} + I_{\text{ion}} + I_{\text{ep}} \quad \text{on } S \quad (3)$$

where \mathbf{n} is the unit vector normal to the membrane's surface, σ_i and σ_e are the intracellular and extracellular conductivities, C_m is the specific membrane capacitance per unit area,

$V_m = \Phi_i - \Phi_e$ is the transmembrane potential, t is time, I_{ion} is the ionic current, and I_{ep} is the current due to electroporation.

For a cell suspended in an electric field, the change in transmembrane potential is

$$V_m = \lambda f a E_o \cos \theta \left[\exp \left(\frac{-t}{\tau} \right) - 1 \right] \quad (4)$$

$$\tau = f a C_m \left(\frac{1}{\sigma_i} + \frac{1}{2\sigma_e} \right) \quad (5)$$

$$f = \left[1 + a G_m \left(\frac{1}{\sigma_i} + \frac{1}{\sigma_e} \right) \right]^{-1} \quad (6)$$

where a is the cell radius, C_m is the membrane capacitance per unit area, λ is a shape factor, G_m is the membrane conductance per unit area, and θ is the angle between the applied electric field E_o and the site on the cell membrane at which the potential is determined [2]. See Figure 1. For most cases, the transient terms can be neglected because the electroporation pulse is much larger than the membrane charging time. If you simplify this further by assuming that the cell is spherical with a shape factor of 1.5, the equation reduces to

$$V_m = 1.5 a E_o \cos \theta \quad (7)$$

If the transmembrane potential exceeds a critical value, i.e., the breakdown voltage, the membrane undergoes irreversible electroporation, which leads finally to the rupture of biological membranes. For most biomembranes, a transmembrane potential of 1 V is sufficient to induce irreversible membrane breakdown and thus kill the cells [41]. It should be noticed that according to Eq. (7), the transmembrane potential is proportional to cell size, which suggests that among a population of cells, the same electrical field may cause different transmembrane potentials. This sheds light on why most current electroporation techniques that work on batches of cells have to trade off between transfection rate and cell viability. It should be also noted that these equations do not include the resting potential of the membrane, which is of the order of 100 mV, negative inside the cell. The transmembrane potential can be measured by staining the membrane with a voltage-sensitive fluorescent dye and recording it using digital video microscopy [4]. One must be careful not to permanently damage the cell via cell lysis. This is difficult since, as the above equation suggests, the transmembrane potential is not uniform and is actually maximum at the poles.

MICROELECTROPORATION TECHNOLOGY

Currently, electroporation is normally done in batches of cells between electrodes and in organisms with electrodes inserted in the tissue. There is little control over the permeabilization of individual cells. Therefore, it is very difficult to study the fundamental biophysics of cell membrane electroporation, and to design optimal and reversible electroporation protocols. As discussed above, micro aqueous pores with diameters of tens to hundreds of angstroms are believed to form in the cell membrane due to the electrical field-induced structural rearrangement of the lipid bilayer. It occurred to us that if electroporation induces pores in the cell membrane then, in a state of electroporation, the current that flows through the cell should be measurable, thereby making electroporation controllable. From this idea, we have developed a new microelectroporation technology

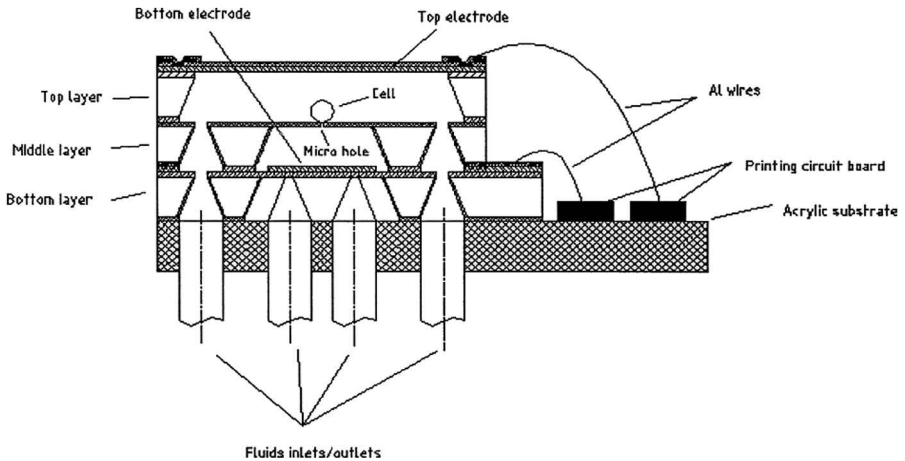


Figure 2. Schematic of a microelectroporation chip in cross section.

that employs a “bionic” chip to study and control the electroporation process in individual cells. The microelectroporation chips are designed and fabricated using standard silicon microfabrication technology. Figure 2 shows a schematic of the chip. Each chip is a three-layer device that consists of two translucent poly-silicon electrodes and a silicon nitride membrane, which together form two fluid chambers. The two chambers are interconnected only through a micro hole ($2\text{--}4\ \mu\text{m}$) on a $1\text{-}\mu\text{m}$ -thick dielectric silicon nitride membrane. In a typical process, the two chambers are filled with conductive solutions and one chamber contains biological cells. Individual cells can be captured in the micro hole and thus incorporated in the electrical circuit between the two electrodes of the chip. Figure 3 shows a cell captured in the hole. When the potential between the electrodes is too low to produce electroporation, the cell is in its normal state, no current flows through the insulating lipid bilayer, and consequently between the electrodes. However, when the electrical potential across the electrodes is sufficient to induce electroporation, a measurable current will flow through the pores of the cell membrane and between the electrodes. Measuring currents through the bionic chip provides important information

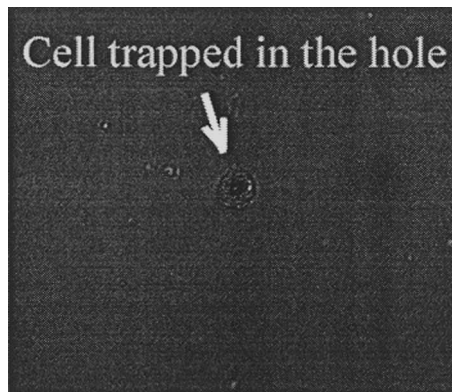


Figure 3. A human prostate adenocarcinoma cell (ND-1) being trapped in the micro hole.

about the membrane electroporation process. Figure 4 shows the currents through the microchip loaded with three human prostate adenocarcinoma cells (ND-1), as a function of the strength of square pulses applied across the electrodes. It illustrates typical behavior of a cell in the chip and shows that the cell behaves electrically somewhat like a Zenner diode, with no current at potentials that do not induce electroporation and currents at potentials that induce electroporation. It is worthwhile to remark that one of the most critical electroporation parameters, i.e., irreversible membrane breakdown voltage, can be quantitatively determined from the current–voltage curve. When the applied pulse strength exceeds the critical one, membrane rupture is triggered and the cell membrane experiences a dramatic increase in conductance, which is reflected as a sudden jump in the currents through the bionic chip. From Figure 4, the breakdown voltages for these specific cells loaded in the chip are between 30 and 35 V.

The work leads to two items. The first item is the proof of our hypothesis: if electroporation produces aqueous channels in the membrane, then electroporated cells should also carry electrical currents, and otherwise should not. This makes the process of electroporation instantaneously detectable when incorporated in an electrical circuit. This is a universal phenomenon, which can occur when the cells are outside or inside the body. Electroporation has been used in the past for the introduction of gene constructs into cells in suspension and into cells inside the body, for production of transgenic organisms, and for treatment of genetic diseases. However, there was no immediate feedback for knowing if the cell was actually electroporated in response to the electrical potential. Now an electrical current measurement provides this information. The second item is the actual accomplishment of producing the chip. The chip has immediate industrial application in introducing macromolecules in cells. With the ability to manipulate individual cells and detect the electrical potentials that induce electroporation in each cell, the chip can be

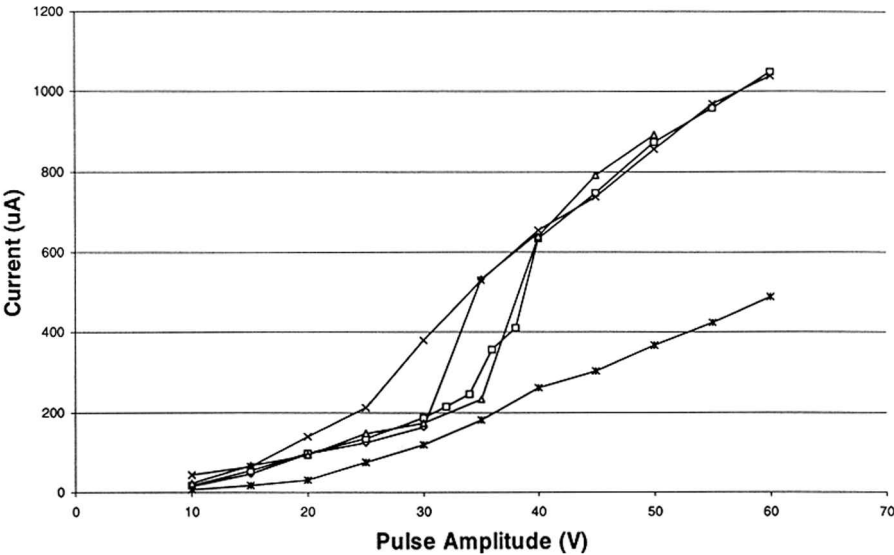


Figure 4. Current though the microelectroporation chip as a function of voltage pulse amplitude. ND-1 cells, pulse duration 60 ms, hole diameter 5 μ m, distance between electrodes 900 μ m. Top curve for open hole, middle curve for cell plugging the hole, bottom curve for closed hole (stray current).

used to study the fundamental biophysics of membrane electroporabilization at the cellular level and in biotechnology for controlled introduction of macromolecules, such as gene constructs, into individual cells.

FUTURE OF ELECTROPORATION

There are many exciting areas for future research. A better understanding of the fundamentals of membrane electroporation and electrode delivery of macromolecules is needed. Work needs to be done to understand what are the fundamental mechanisms involved in the mass transfer across the cell membrane during electroporation. We hope that the microelectroporation device which we have built could be used to generate this understanding. Electroporation of tissues in vivo is most likely to become an important area of research. There is as yet no understanding of how to treat whole tissues where there is such a large variation in tissue properties and such stringent parameters to permeate a cell. There is an obvious need to actively monitor and control the voltage being applied to the tissue. One way is by studying individual cells and from that developing predictive models for in-vivo tissue electroporation. In another method, the idea that electrical current measurement can be used to control electroporation, employed in our microelectroporation device, could be also expanded to electroporation in tissue. It is easy to envision that, in the future, genetic treatment of human diseases or treatment of cancer will be individually designed and produced under real-time electronic control. We hope that the new technology for correlating voltage and current described in this article is another step toward seeing this vision become a reality.

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